

The University of Chicago

A STUDY OF SOME OF THE FACTORS
PROMOTING DISSOCIATION OF
BACTERIUM DYSENTERIAE, SONNE

A PART OF A DISSERTATION SUBMITTED TO
THE FACULTY OF THE DIVISION OF THE
BIOLOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF HYGIENE AND
BACTERIOLOGY

1933

By
ROBERT BARTON DIENST

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A STUDY OF SOME OF THE FACTORS PROMOTING DISSOCIATION OF BACTERIUM DYSENTERIAE, SONNE

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Variation in colony form has been mentioned in several of the studies of the Sonne type of *Bacterium dysenteriae*. In his original publication Sonne (1915) pointed out that colony variants were encountered more frequently among his "type III" cultures than in those of other dysentery bacilli. Thjøtta (1919) also noted this variation in his study of similar cultures isolated in Norway and Thjøtta and Waaler (1932) have reported that both S and R colonies may be obtained on first culture from the stool. Ørskov and Larsen (1925) described four colony variants, two of which possessed biochemical properties different from the original culture. More recently the Sonne type was used by Koser and Styron (1930) in a systematic study of the influence of environment in the production of variant colonies.

The work to be reported here was undertaken as a further study on the effects of environmental influence in promoting variation. It was hoped that continued study along this line might afford some insight into the underlying factors responsible for those changes which so often seem to be a matter of chance. Also, it was thought that any means which might be found for regularly and quickly producing variant colonies would be a distinct aid in the further study of these changes.

In the first part of the work, the effect of different concentrations of the ingredients of ordinary nutrient broth was studied. In later work other conditions of growth were tried: casein-digest medium, different concentrations of tryptophane in nutrient broth, a meat medium and, in contrast to this, several synthetic

media in which the organism grew with difficulty. The effect of filtrates of homologous and heterologous strains and the effect of both agglutinating serum and normal rabbit serum were also tried.

METHODS

Smooth cultures were employed for the most part. Each laboratory culture used for these experiments was carried through at least ten reisolations on agar plates. Without exception, all colonies that developed on the tenth transfer were smooth and, in many instances, all colonies that appeared throughout the entire series of platings were smooth. These smooth isolations were then inoculated into the various media. All media were adjusted so that a pH 7.0 to 7.2 would be obtained after sterilization. In most of the following experiments two strains of the Sonne type were used. These were selected as being representative of twelve strains previously studied in aging cultures of nutrient broth.

After inoculation of the various media the cultures were allowed to age, usually at 37°C., for the purpose of determining the effect of such a procedure as contrasted with the more common method of serial transplants. At different intervals samples were withdrawn and streaked over the surface of agar plates so that well isolated colonies were obtained. Before the samples were taken the tubes were thoroughly agitated so that a fair sample might be obtained. After twenty-four hours' incubation at 37°C., the colony growth on these plates was studied with a binocular microscope ($\times 30$ magnification). One hundred colonies were counted whenever possible and the results recorded in percentage of each type of colony. It should be emphasized here that whatever the liquid medium in which the organisms were grown, ordinary nutrient agar was always used for plating.

The variations in colony form encountered throughout this work were similar for the most part to those previously described by Arkwright (1921) for the colon-typhoid-dysentery group. The smooth, intermediate and rough forms of *Bact. dysenteriae* Sonne have been described by Koser and Styron (1930) and no

additional description is needed here. In addition to these forms, two strains of "small colony" variants, similar in many respects to the G forms reported by Hadley (1931), were found.

Effect of different concentrations of peptone and beef extract upon dissociation

Koser and Styron (1930) reported that when a culture of *Bact. dysenteriae* Sonne was grown in different concentrations of peptone, the greatest departure from the smooth type occurred in the 5 per cent peptone solution. A concentration of 20 per cent was much less effective in stimulating change and in 0.5 and 1 per cent peptone there was little variation from the S form even after prolonged incubation. De Kruif (1922), however, found that in high concentration of peptone (20 per cent), "mutation" of the bacillus of rabbit septicemia was rapid and colony variants might constitute 90 per cent of the total within five to six days.

The first experiment was planned to show the effect of different concentrations of both peptone and beef-extract together upon the production of variant colonies. The following concentrations were inoculated with the S colony form of Sonne 268 and 269:

PEPTONE (BACTO)	BEEF EXTRACT (SWIFT)	CONCENTRATION
<i>per cent</i>	<i>per cent</i>	
0.3	0.1	$\frac{1}{3}$ that of nutrient broth
1.0	0.3	Ordinary nutrient broth
3.0	0.9	3 times that of nutrient broth
5.0	1.5	5 times that of nutrient broth
10.0	3.0	10 times that of nutrient broth

Variant colony forms were noted on plates prepared from aging cultures in all of the above media, although changes from the smooth form occurred rather slowly. Usually several weeks elapsed before the changes had progressed beyond the Sr stage.¹ With further aging of the culture small proportions (5 to 15 per cent) of sR colonies appeared and finally true R forms. At times

¹ Throughout this paper Sr signifies intermediate forms closely resembling true smooth forms while sR designates intermediate forms more closely resembling rough forms.

the R type comprised 80 to 90 per cent of the colonies which appeared after several months of aging.

Effect of different concentrations of sodium chloride in nutrient broth

Tubes containing 0.5, 2.0 and 5.0 per cent concentrations of salt in nutrient broth were inoculated with Sonne 268 S and 269 S. These cultures were incubated at 37°C., allowed to age and plated as in the preceding experiment. Variation in colony form seemed to be slightly more noticeable after growth in the 0.5 per cent concentration of salt than in the higher concentrations. However, not even in this concentration did R colonies appear and only the sR stage was reached after thirty to forty-two days' incubation in the 0.5 per cent salt. In some cases the sR colonies were produced in 2 per cent salt but apparently reverted to the Sr colony form or disappeared upon continued incubation. The higher concentration of the salt (5 per cent) killed the organisms after a month of incubation.

When the medium was first inoculated with the S colony form, the organisms grew in the form of flocculent sediment in the 2 and 5 per cent concentrations of the salt while the rest of the medium was perfectly clear. There was also a surface growth which settled to the bottom when disturbed. In these concentrations of the salt, the growth appeared similar to that produced when an R colony form was inoculated into nutrient broth. When agar plates were streaked from these media, however, the colonies which developed were not the R type but were S and Sr colony forms.

Effect of growth in various media

Beef heart medium. Tubes containing 10 cc. of beef-heart medium were inoculated with the S form of Sonne 268 and 269. These cultures were grown and examined in the usual manner. This experiment was performed twice. On each occasion there appeared to be very little difference between the effect of this "rich" medium and the effect of ordinary nutrient broth upon the change in colony forms. A small percentage (10 to 20) of sR colonies were observed after the cultures had aged in the beef-heart medium for thirty-five days; no rough colonies were seen at any time during the experiments.

Synthetic media. Several combinations were prepared so that each medium contained 0.05 per cent dipotassium phosphate and 0.05 per cent magnesium sulfate. To one portion of this mixture 0.3 per cent aspartic acid was added; to other portions either 0.3 per cent asparagin, 0.3 per cent asparagin plus 3 per cent glycerol, 0.3 per cent ammonium succinate or 0.3 per cent glyco-coll was added. The media were adjusted to a pH 7.0. Each medium was perfectly clear after being sterilized in the autoclave. Test tubes containing 8 cc. of each medium were inoculated with the same two strains of the Sonne type which had been used in the previous experiments.

Growth in these synthetic media was not accompanied by any unusual production of rough colonies or other variant forms. Throughout the period of incubation for thirty-eight days chiefly S and Sr colony forms were seen. Only in the medium containing asparagin plus glycerol was there a tendency for the Sonne strains to become rough. In this medium 20 per cent sR colony forms were observed after the cultures had been incubated for thirty-eight days. Thus, media which supplied only limited kinds of foodstuffs exerted no more effect than nutrient broth in promoting the S to R colony changes.

Casein-digest media. Casein-digest medium was prepared by the method of Kulp and Rettger (1924) in which a sodium bicarbonate solution of casein is digested by trypsin. Also, a "tryptophane" broth was prepared by making a 1 per cent solution of commercial dehydrated casein digest powder.² Both media were then inoculated with the S colony forms of the Sonne strains and allowed to age at 37°C. Table 1 shows that changes in colony form and eventual production of R colonies were more pronounced under these conditions than in any of those previously studied. This experiment was repeated, using several different strains of the Sonne type, with practically the same results.

In still another experiment with 2 per cent casein-digest medium, colonies of the G type (Hadley 1931) were found after thirty days' incubation at 37°C. This culture was apparently sterile when spread on agar plates after two weeks, but after

² Prepared by Digestive Ferments Company.

thirty days' incubation, when several loopfuls of this apparently sterile culture were streaked over nutrient agar plates, G colonies appeared in twenty-four hours. These plates seemed sterile when studied with the naked eye, but upon examination with the binocular microscope numerous tiny colonies could be seen along the line of streak.

TABLE 1

Showing the types of colonies developing on agar plates when streaked from aging cultures in casein-digest medium and commercial "tryptophane" broth

DAYS INCUBATED	TRYPTOPHANE BROTH		CASEIN-DIGEST MEDIUM	
	Sonne 268 S	Sonne 269 S	Sonne 268 S	Sonne 269 S
1	100% S	100% S	100% S	100% S
7	25% S 75% Sr	10% S 90% Sr	30% S 70% Sr	45% S 55% Sr
17	25% S 75% Sr	10% S 85% Sr 5% sR	30% S 70% Sr	10% S 90% Sr
24	10% S 90% Sr	90% Sr 10% sR	40% Sr 20% sR 40% R	35% Sr 35% sR 30% R
31	100% Sr	85% Sr 10% sR 5% R	20% Sr 20% sR 60% R	30% Sr 20% sR 50% R
40	100% Sr	15% Sr 10% sR 75% R		

Nutrient broth containing pure tryptophane. In the above experiments it was thought that perhaps the presence of an increased amount of the amino acid, tryptophane, was responsible for the greater production of colony variants. Different concentrations of pure tryptophane were made in 1 per cent peptone water and adjusted to pH 7.0. The following concentrations of tryptophane were used: 0.05, 0.1, 0.3, 0.5, 0.8 and 1 per cent. These tryptophane-containing tubes were then inoculated with the smooth colony form of Sonne 268 and 269 and allowed to

age at 37°C. The amount of colony variation in these media was not markedly different from that observed in nutrient broth alone. Only a very small number of sR colony forms was observed. No rough forms were noted on any of the plates streaked from cultures aged for forty days, whereas 60 to 90 per cent rough colonies were produced when Sonne 268 S was aged in casein-digest medium for forty days. Evidently the tryptophane itself had very little effect in the production of the greater amount of variation previously found in the casein-digest medium or the commercial "tryptophane" broth.

Effect of metabolic products in causing variation of colony form

It was observed in previous experiments that the S to R changes in colony form were enhanced by cultivation in a casein-digest medium. If the formation of variant colonies is due to the accumulation of metabolic products in such a medium, then an homologous filtrate of a culture showing decided variation in colony forms should further enhance this S to R change. It might also provide an easy means of obtaining R or other variants when these forms are desired for experimental study.

Filtrates were prepared in the following manner: Sonne strains 268 S, 269 S, 268 R and 269 R were each inoculated into different large test tubes containing 30 cc. of the casein-digest medium. These were incubated at 37°C. for ten days. Samples were then withdrawn from each of the tubes and plated on nutrient agar. The proportion of different colony types found on the agar plates was:

268 S, 10 per cent S	and 90 per cent Sr
269 S, 10 per cent S	and 90 per cent Sr
268 R, 20 per cent sR	and 80 per cent R
269 R, 10 per cent sR	and 90 per cent R

These cultures were then filtered through Seitz-Werke filters with a single filter pad at 5 inches vacuum until about 25 cc. of the medium had passed through the filter. The filtrates were tested for sterility by incubation at 37°C. and by inoculation of nutrient broth tubes. After testing for sterility, each filtrate was inoculated with its homologous strain and incubated at 37°C.

By aging a *smooth* colony form in its homologous filtrate, 20 to 25 per cent R colony forms were obtained in fourteen days. At this time no smooth colonies could be seen. The remaining colonies were intermediate between smooth and rough in appearance. In contrast to this, it was interesting to see that a filtrate prepared from a ten-day growth of the *rough* form in this casein-digest medium did not enhance the S to R changes when an S culture was inoculated into it.

Effect of immune rabbit serum upon change in colony form

A number of workers have reported that homologous immune serum in nutrient broth stimulates the production of colony variants. Griffith (1923) was able to produce R colony forms of pneumococci by growth of an S culture in homologous immune serum. Dulaney (1928) readily produced S to R variation of *Bact. coli-communis* by cultivation of the S form in homologous immune serum. She was able to produce S colony forms by cultivation of R forms in homologous R immune serum. Soule (1928), in his work with *B. subtilis*, found that when R and S forms of cultures were placed in the S immune serum, no change occurred in R but the S culture produced 80 per cent R colony forms at the end of twenty-four hours. He also found that the use of 10 per cent immune serum in broth was just as effective as any higher concentration. Larkum (1928) reported that growth of R types of *Bact. typhosum* in S immune serum resulted in the production of S colony forms and that the tendency of S types to dissociate to R forms was inhibited by such serum. Nungester (1929) obtained R types of *B. antracis* by aging the S colony form in normal serum (10 per cent) but similar results were obtained by aging in plain broth.

The following experiments were performed to test the effect of immune serum upon the organisms used in this study. Four rabbits were used to prepare agglutinating sera with the S and R forms of cultures 268 and 269. The rabbits were injected twice weekly for three weeks; first, subcutaneously with heat-killed organisms and, finally, intravenously with the living organisms. One cubic centimeter amounts of a saline suspension were injected

each time. The rabbit inoculated with the 268 R culture showed a titer of only 1:10 while rather high titers were produced in the other 3 rabbits. A titer of 1:1280 was obtained from each of the 268 S and 269 S strains. The 269 R colony form produced a titer of 1:640. These animals were bled by cardiac puncture and the serum collected aseptically. Each sterile agglutinating serum was added to a tube of nutrient broth so that a 10 per cent concentration of the serum was obtained in 5 cc. of broth. The tubes were then incubated and tested for sterility before being inoculated with the different strains of Sonne types. Nutrient broth tubes containing 10 per cent normal rabbit serum were used for controls.

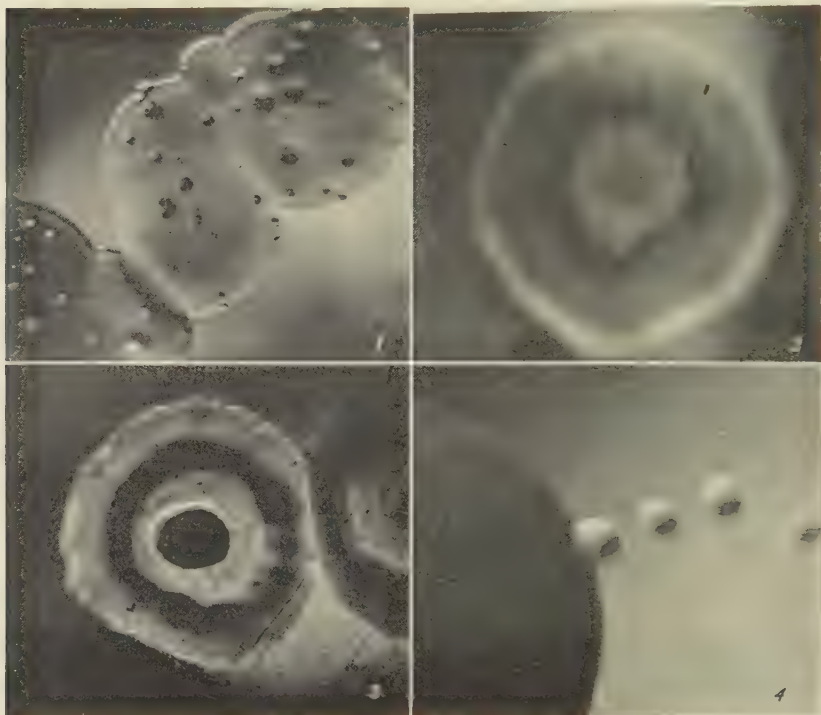
It was found that the S type when inoculated into 10 per cent homologous agglutinating serum did not show any marked tendency to change to the R colony form throughout a period of seventy-four days. Indeed there seemed to be less tendency to produce variant colonies in the presence of the serum. There was very little difference between the effect of homologous, non-homologous and normal rabbit serum upon variation in colony form. In some further work, it was found that R types were not reverted to S types by cultivation in either homologous R serum, non-homologous R serum or normal rabbit serum.

MISCELLANEOUS OBSERVATIONS

Secondary colonies. Colonies showing the peculiar secondary growth described by many workers as "daughter colonies" were observed many times during the different experiments, especially when agar plates were allowed to stand for a time at room temperature. These daughter colonies seemed to develop chiefly in the smooth and the intermediate colony forms. Daughter colonies were never observed to develop in the rough colony form. Figure 1 shows that usually the daughter colonies were scattered throughout the old colony.

Erosive phenomenon. In attempting to obtain "G" colony forms by aging cultures in 1 per cent casein-digest medium, an unusual colony form was obtained from one of the Sonne strains as shown in figure 2. These colonies were observed on a nutrient

agar plate streaked from a casein-digest culture after it had incubated for seven days at 37°C. Some of the colonies appearing on the plate were as large as the usual smooth colony but had a decided depression in the center. The diameter of this area increased slowly upon continued incubation, as though the central



FIGS. 1 TO 4

FIG. 1. Daughter colonies of strain 2053 (magnification $\times 10$).

FIG. 2. Colony obtained from strain 2053 showing central "lysis." The lens was focused on the lysed area (magnification $\times 100$).

FIG. 3. Colony obtained from strain 2053 showing secondary growth in "lysed" area (magnification $\times 200$).

FIG. 4. The G colony form of strain 2053 and a section of a normal smooth colony (magnification $\times 100$).

portion were being gradually digested. If an agar plate was streaked with cells from the center of the "lysed area," colonies grew in twenty-four hours which were rather flat and spreading with a clear watery periphery. If an agar plate was streaked

with some of the cells from the edge of the "lysed area," typical smooth colonies developed in twenty-four hours. When a portion of the "lysed area" was mounted on a slide and stained by Gram's method it was observed that most of the cells were only lightly stained and appeared to be disintegrating, though a few presented the normal deep pink appearance. Secondary growth as shown in figure 3 sometimes developed in the digested area of the old colony. When this secondary growth was transplanted to a nutrient agar plate, typical smooth colonies developed in twenty-four hours.

Preisiz (1904) observed this gradual disintegration of an old colony while studying the anthrax bacillus. He described the reaction as an "erosive phenomenon." Hadley (1924) has also made similar observations while studying transmissible lysis in *B. pyocyaneus*.

A study of the filtrates of cultures of Bact. dysenteriae, Sonne

In connection with the experiments mentioned in the foregoing sections a number of filtrations were made. This was done with the view that, if a filtrable stage does exist, the possibility of demonstrating it might be greater at a time when the culture is undergoing those changes which give rise to differences in colony form and cell morphology. A number of cultures in various liquid media were allowed to age until they showed evidence of change in colony form when spread over agar plates. They were then filtered. In other cases, instead of using cultures at the time when colony variants first appeared, filtrations were made with several-months old cultures which had shown some variation in colony form for a considerable time.

Method

Seitz-Werke and Berkefeld filters were used in all the experiments. The Berkefeld filters were first tested by the use of a twenty-four-hour growth of *Chromobacterium prodigiosum*. Great care was taken to eliminate any possible contamination during the process of filtration and subculturing of the filtrate. The filtrates were studied by subculturing 1 cc. amounts in dif-

TABLE 2
Results of experiments on filtration

STRAIN	MEDIUM	AGE WHEN FIL- TERED	COLONY FORMS WHEN FILTERED	FILTERS USED	VACUUM USED	TESTS ON FILTRATES					
						Filtrate alone	Nutrient broth	Glucose veal infusion	Beef heart	Lactose agar	Nutrient agar plates
268	5x broth	days 1	100% S	Seitz	inches 6	— (30)	— (30)				— (15)
269	5x broth	1	100% S	Seitz	6	— (30)	— (30)				— (15)
268	5x broth	15	10% S 80% Sr 10% sR	Seitz	20	— (35)	— (35)	— (35)	— (35)		— (15)
269	5x broth	15	10% S 85% Sr 5% sR	Seitz	20	— (35)	— (35)	— (35)	— (35)		— (15)
268	Casein- digest	20	60% Sr 40% sR	Seitz	20	— (30)	— (30)	— (30)	— (30)	— (20)	— (20)
269	Casein- digest	20	55% Sr 45% sR	Seitz	20	— (30)	— (30)	— (30)	— (30)	— (20)	— (20)
2053	Casein- digest	50	25% Sr 45% sR 30% R	Berkefeld N Berkefeld W Seitz	5 5 5	— (90) — (90) — (90)		— (90) — (90) — (90)		— (7) — (7) — (7)	
Brown	Casein- digest	50	10% S 50% Sr 30% sR 10% R	Berkefeld N Berkefeld W Seitz	5 5 5	— (90) — (90) — (90)		— (90) — (90) — (90)		— (7) — (7) — (7)	

St. Andrews	Casein-digest	50	55% Sr 30% sR 15% R	Berkefeld N Berkefeld W Seitz	5				Liver infusion broth	
268	Casein-digest	50	30% S 60% Sr 10% sR	Berkefeld N Berkefeld W Seitz	5	— (90) — (90) — (90)	— (90) — (90) — (90)	— (7) — (7) — (7)		
Brown	Casein-digest	112	60% S 40% Sr	Berkefeld N Berkefeld W Seitz	5	— (28) — (28) — (28)	— (28) — (28) — (28)	— (4)* — (4)* — (4)*		
Brown	Casein-digest	300	90% Sr 10% sR	Berkefeld N Seitz	5	— (100)† — (100)†	— (50) — (50)	— (20)* — (20)*		
268	Casein-digest	300	5% Sr 95% sR	Berkefeld N Seitz	5	— (100)† — (100)†	— (50) — (50)	— (20)* — (20)*		
269	Casein-digest	300	60% Sr 40% sR	Berkefeld N Seitz	5	— (100)† — (100)†	— (50) — (50)	— (20)* — (20)*		
269	Casein-digest	300	20% Sr 80% sR	Berkefeld N Seitz	5	— (35)† — (35)†	— (35) — (35)	— (20)* — (20)*		

Negative results in the filtrates and the subcultures are shown by the — sign. The figures in parentheses indicate the number of days observed.

* Serial transplanting as described by Hauduroy.

† Ten cubic centimeters of filtrate also placed in a sterile tube and sealed. This tube was then held at room temperature for six months. All subcultures made at the end of six months showed no growth.

ferent liquid media and by allowing 10 cc. amounts of the filtrate to incubate at room temperature for a number of days. Some of the filtrates were cultured by serial transplanting on lactose agar as described by Hauduroy (1927). In some cases 1 cc. of the filtrate was flooded over an agar plate and incubated at 37°C. for several days. Before discarding any of the filtrates or subcultures of the filtrates in which there was apparently no growth, samples were withdrawn and again subcultured in nutrient broth and on agar plates. All agar plates were examined under the binocular microscope for growth.

Table 2 gives the tabulated results of these experiments. Twenty-nine filtrates were examined and all of them were apparently sterile. There was no evidence of gradual development from a presumably filtrable stage to the usual form when filtrates were subcultured in media most favorable for growth of the laboratory strains used.

SUMMARY

A study was made of some of the factors promoting dissociation of *Bact. dysenteriae* Sonne when grown in various liquid media.

Variant colony forms were obtained by growing the organisms in different concentrations of peptone and beef extract. The concentrations containing 3 and 5 times the amount of peptone and beef extract in nutrient broth promoted the S to R changes, though true R forms appeared only after the cultures had aged from forty-two to ninety days at 37°C. Changes in colony form were less marked in the other concentrations.

Different concentrations of sodium chloride in nutrient broth did not enhance dissociation. Variant colony forms were not produced in a "rich" medium (beef heart) or in a "poor" medium (synthetic) to any greater extent than in ordinary nutrient broth.

Smooth cultures when inoculated into 10 per cent homologous agglutinating serum did not show any marked tendency to change to the rough colony form, even after being incubated at room temperature for seventy-four days. There was no difference between the effect of homologous, non-homologous and normal rabbit serum.

Daughter colonies were often noted when studying colonies aged from seven to fourteen days on nutrient agar plates.

The G colony form as produced by *Bact. dysenteriae* Sonne is apparently a rare colony form. It was observed only twice out of 48 cultures studied and these small colony forms were obtained only after plating out old cultures.

The erosive phenomenon of Preisz was observed with one strain.

A filtrable form of *Bact. dysenteriae* Sonne could not be demonstrated by the filtration of cultures in which variation was taking place, as shown by the formation of different forms of colonies on agar plates. Berkefeld N, W and Seitz filters were used. Filtrates were subcultured in various media and Hauduroy's technic of serial transfer on lactose agar plates was also used. All subcultures were negative and no evidence could be found of a gradual development or "growing out" of a filtrable form.

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